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DNA vaccine encoding type IV pilin of *Actinobacillus pleuropneumoniae* induces strong immune response but confers limited protective efficacy against serotype 2 challenge

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ABSTRACT

Actinobacillus pleuropneumoniae is a gram-negative bacterial pathogen that causes swine pleuropneumonia, a highly contagious and often fatal disease that occurs worldwide. Our previous study showed that DNA vaccines encoding Apx exotoxin structural proteins ApxIA and/or ApxIIA, are a promising novel approach for immunization against the lethal challenge of *A. pleuropneumoniae* serotype 1. Vaccination against *A. pleuropneumoniae* is impeded by the lack of vaccines inducing reliable cross-serotype protection. Type IV fimbrial protein ApfA has been shown to be present and highly conserved in various serotypes of *A. pleuropneumoniae*. A novel DNA vaccine encoding ApfA (pcDNA-*apf*A) was constructed to evaluate the protective efficacy against infection with *A. pleuropneumoniae* serotype 2. A significant antibody response against pilin was generated following pcDNA-*apf*A immunization, suggesting that it was expressed *in vivo*. The IgG subclass (IgG1 and IgG2a) analysis indicates that the pcDNA-*apf*A vaccine induces both Th1 and Th2 immune responses. The IgA analysis shows that mucosal immunity could be enhanced by this DNA vaccine. Nevertheless, the strong antibody response induced by pcDNA-*apf*A vaccine only provided limited 30% protective efficacy against the serotype 2 challenge. These results in this study do not coincide with that the utility of type IV pilin is a good vaccine candidate against other infectious pathogens. It indicates that pilin should play a limited role in the development of a vaccine against A. pleuropneumoniae infection.

1. Introduction

Actinobacillus pleuropneumoniae, a bacterial pathogen belonging to the family *Pasteurellaceae*, is the etiological agent of porcine pleuropneumonia that causes severe respiratory disease in swine [1]. The pathogen is transmitted by respiratory droplets or direct contact with infected pigs, and it adheres and colonizes on the swine lower respiratory epithelial cells [2,3]. Colonized animals can act as asymptomatic carriers, spreading the pathogen to previously unexposed herds [4]. On the basis of the presence of capsular polysaccharide antigens, 15 serotypes have been reported, which have regionally variable prevalence and distribution [5,6]. All serotypes are capable of causing disease, although there are evidences that the virulence varies in different serotypes [7,8]. The virulence of *A. pleuropneumoniae* is caused by multiple factors: capsular polysaccharide [9,10], lipopolysaccharide (LPS) [11], outer membrane proteins [12,13], iron-acquisition systems [14,15], adhesins [1], and Apx toxins [16] have been reported as putative virulence elements of this pathogen.

The diversity among the 15 serotypes of *A. pleuropneumoniae* has made effective vaccination difficult; inactivated whole-cell vaccines usually only confer protection against the homologous serotype [17]. Most of the current commercial vaccines against *A. pleuropneumoniae* contain chemically inactivated bacterins of 3 or 4 serotypes; these commercial bacterins afford only partial protection by decreasing mortality but not morbidity. The Porcilis APP^{TM} (Intervet) subunit vaccine, composed of ApxI, ApxII, ApxIII toxoids, and a 42-kDa outer membrane protein, is considered effective at preventing acute infection. However, it does not prevent bacterial colonization and is not protective against all serotypes of *A. pleuropneumoniae* [18,19]. A rational approach to improve the vaccine efficacy is to look for novel antigen candidates with high immunogenicity and protective efficacy for all serotypes. Type IV pilin of *A. pleuropneumoniae* might be suitable for this purpose.

Fimbriae are common mediators of bacterial adherence to host mucosal epithelial cells. Type 4 fimbriae are produced by

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many species of pathogenic gram-negative bacteria [20]. Fimbriae are usually highly antigenic and several fimbrial antigens have been used successfully as subunit vaccine candidates against various bacterial pathogens including *Moraxella bovis* [21], *Dichelobacter nodosus* [22], *Proteus mirabilis* [23], and enterotoxigenic *Escherichia coli* [24]. In contrast, the fimbrial protein of *Burkholderia mallei* is highly immunogenic but fails to protect against lethal aerosol challenge in mice [25]. Currently no *A. pleuropneumoniae* vaccine provides protection against all serotypes of this pathogen [26]. The type 4 fimbrial genes of *A. pleuropneumoniae* have been identified and the fimbria structural protein gene, *apfA*, is highly conserved in all serotypes [27]. It has been recently shown that the recombinant ApfA may interfere with immunity when it is mixed in a multicomponent recombinant vaccine [28]. However, the role that ApfA itself plays in conferring protective efficacy is still unclear. Therefore, the potential of fimbrial protein ApfA from *A. pleuropneumoniae* as a vaccine candidate is worthy of investigation.

In a previous study, we developed a DNA vaccine encoding ApxIA and ApxIIA, which seems to be an exciting new approach with great promise for vaccine development against *A. pleuropneumoniae* serotype 1 lethal challenge [29]. However, the heterogeneity of 15 serovars limits the vaccine efficacy afforded by a single antigen. Several studies have mainly focused on finding novel antigens that are highly conserved among various serotypes or even in all serotypes [30,31]. For these reasons, we set out to discover novel antigens to develop multivalent vaccine to enhance protective efficacy against infections with a variety of serovars. In this study, the immunogenicity and protective efficacy of a DNA vaccine encoding ApfA, type IV pilin of *A. pleuropneumoniae*, were evaluated in mice via immunization and challenge experiments to determine whether the vaccine was capable of providing protection against a serotype 2 challenge.

2. Materials and methods

2.1. Bacteria strains and growth conditions

The *A. pleuropneumoniae* serotype 2 (Denmark strain) was a kind gift from Animal Health Research Institute, Council of Agriculture (Tansui, Taiwan). The bacteria were cultured in brain heart infusion medium (Bacto), supplemented with 0.1% β-nicotinamide adenine dinucleotide (Sigma) at 37 °C in a shaking incubator at 180 rpm. *E. coli* TOP10 (Invitrogen) served as a host for DNA manipulation experiments. The *E. coli* cultures were routinely grown at 37 °C in Luria–Bertani (LB) broth or agar (15 g/l) and were supplemented with ampicillin (50 μg/ml), where appropriate, for the selection of recombinant plasmid-containing strains.

2.2. Construction of the pcDNA-apfA DNA vaccine

The genomic DNA of *A. pleuropneumoniae* serotype 2 was extracted using the Genomic DNA Mini Kit (Geneaid, Taiwan) and used as a template for PCR amplification of the target sequences. *apf*A was amplified with PfuUltra II DNA polymerase (Agilent Technologies) and a primer pair was designed based on the sequence of GenBank accession number AY235718; the primer pair sequences are shown in Fig. 1A. The reactions were performed in a PCR machine (ASTEC, PC320, Japan) programmed as follows: 94 °C for 2 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1.5 min and an extension step at 72 °C for 10 min at the end of the cycles. The amplified products were recovered with the Gene-Spin™ 1-4-3 DNA Purification Kit (Protech, Taiwan); then they were cloned into the pcDNA3.1 Directional TOPO vector (Invitrogen), transformed to *E. coli* TOP10 and the transformants were selected from LB-ampicillin medium. The brief map of the recombinant plasmid is shown in Fig. 1B. Recombinant plasmids were propagated in *E. coli* TOP10 and isolated using High-Speed Plasmid Mini Kit (Geneaid, Taiwan) according to the manufacturer's recommendations. Restriction enzymes *Bam*HI and *Eco*RV were used for the preliminary identification of recombinant plasmids. Cloned *apf*A genes were sequenced using a fluorescence-based sequencer at the Seeing Bioscience Company (Taipei, Taiwan). Sequences were analyzed using the BLAST (Basic Local Alignment Search Tool) software provided by NCBI (National Center for Biotechnology Information) to verify the cloned gene sequences.

2.3. In vitro expression of ApfA by bacterial and mammalian cells

The cloned *apf*A gene-encoded protein could be expressed in prokaryotic and eukaryotic systems because the CMV (cytomegalovirus) and T7 promoters were included in the expression vector (Fig. 1B). The expression of the ApfA protein was first verified in *E. coli* by Western blot analysis and the protein was used for the enzyme-linked immunosorbent assay (ELISA) for detection of antisera responses. The expression of ApfA proteins in eukaryotes was also confirmed in the mouse embryonic fibroblast cell line NIH3T3 (BCRC 60008; Bioresource Collection and Research Center, Taiwan) by Western blot and immunofluorescence assay.

Western blot analysis of ApfA protein expression was performed as previously described [29]. Briefly, the total cell lysates of samples were electrophoresed on 15% SDS–PAGE gels and subsequently transferred to nitrocellulose membrane. After blocking with 5% skim milk powder in TBST (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20), proteins were selectively identified using a 1:5000 dilution of alkaline-phosphatase conjugated mouse anti-V5 antibody (Invitrogen). The color development of blot was monitored and visualized with the BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium) liquid substrate system (Sigma).

ApfA expression in NIH3T3 cells was confirmed by indirect immunofluorescence assay (IFA). The cells were transfected with 0.6 μg of plasmid DNA using GeneJuice Transfection Reagent (Novagen) according to the manufacturer's protocol. Eighteen hours after transfection the medium was aspirated, and the transfected cells were washed and then fixed with 5% formalin in PBS (phosphate buffered saline). After washing 3 times with PBS/1% Triton X-100 (PBS/Triton) the cells were subsequently incubated with 3% bovine serum albumin in PBS at 37 °C for 1 h to block nonspecific binding. After aspirating the blocking solution mouse anti-V5 antibody diluted at 1:500 in PBS was added and incubated at 37 °C for 1 h. The cells were washed and then 300 μl of 1:300 diluted fluorescein-conjugated goat anti-mouse IgG antibody (Kirkegaard & Perry Laboratories, Inc.) was added and incubated in the dark at 37 °C for 1 h. After washing the cells with PBS/Triton 3 times, the cells were examined with a fluorescence microscope (Nikon, Japan).

2.4. Animals and immunization schedule

Six- to 8-week-old female inbred specific pathogen-free (SPF) BALB/c mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan). All animal experiments were performed in accordance with institutional guidelines of National University of Kaohsiung. Animals were allowed to stabilize for 10 days before the experiments to minimize any stress due to transportation and environmental changes affecting the immune system. The DNA vaccines used for immunization were prepared using Winzard Plus Maxipreps DNA Purification System (Promega) according to the manufacturer's instructions. Groups of 10 mice, except for the PBS